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SHORT COMMUNICATION

GUS assay in hypocotyl generated callus of chili (*Capsicum annuum* L.) cultivar Kashi Anmol

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Chili (*Capsicum annuum* L.) is an essential spice crop cultivated extensively worldwide due to its pungency, rich nutritional profile, and economic significance in various industries, including food, pharmaceuticals, and medicine (Nazeer et al., 2023). It serves as a key ingredient in culinary applications, while its bioactive compounds contribute to numerous health benefits. Despite its importance, chili production is frequently challenged by both biotic and abiotic stress factors that adversely affect growth, yield, and quality (Chhapekar et al., 2018). Biotic stresses include a wide range of fungal, bacterial, and viral pathogens, as well as insect pests such as aphids, thrips, and whiteflies, all of which significantly reduce crop productivity (Parisi et al., 2020). These pests and diseases lead to severe yield losses, posing a substantial threat to commercial cultivation. Additionally, abiotic stress factors such as drought, extreme temperatures, and soil salinity further constrain chili production by negatively impacting plant health and fruit development (Khaitov et al., 2019). Addressing these challenges requires continuous efforts in breeding and biotechnological interventions to enhance resistance and improve crop resilience. Kashi Anmol is a very promising chili cultivar developed through recurrent selection (Madhavi et al., 2023). This variety is recognized for its superior agronomic traits, including high fruit yield, rich nutritional content, and adaptability to diverse environmental conditions. Due to its agricultural and economic importance, Kashi Anmol has become a valuable resource for further genetic improvement aimed at increasing resistance to biotic and abiotic stresses. Advanced breeding techniques and biotechnological approaches, including genetic transformation, are being explored to enhance disease resistance, stress tolerance, and overall crop quality in this variety (Kumar et al. 2019).

Prior to germination, the seeds were surface sterilized to eliminate potential contaminants. The sterilization process involved treatment with 70% ethanol for one minute, followed by exposure to sodium hypochlorite for

five minutes. To ensure the complete removal of sterilizing agents, the seeds were thoroughly rinsed with sterile distilled water before being transferred to a growth medium. The seeds were cultivated in magenta boxes containing half-strength Murashige and Skoog (MS) media to promote germination and early seedling development under controlled environmental conditions. The growth conditions were maintained at a temperature of 25 ± 2°C with a 16-hour photoperiod and 60% relative humidity to optimize plant growth. After 12 days of seedling development, hypocotyl segments were excised and cultured on Murashige and Skoog (MS) medium supplemented with thidiazuron (TDZ) at a concentration of 1.0 mg/L, indole-3-acetic acid (IAA) at 0.2 mg/L, and gibberellic acid 0.2 mg/L (GA) to induce callus formation (Kothari et al., 2010). The controlled in vitro conditions provided an ideal environment for studying tissue culture responses, which is crucial for further genetic manipulation and plant improvement. A total of 400 hypocotyl explants of *Capsicum annuum* were cultured for callus induction, out of which 300 successfully developed callus. The pCAMBIA2301 vector carrying the *uidA* gene (encoding *β-glucuronidase*, GUS) under the CaMV 35S promoter and the *nptII* gene for kanamycin resistance was introduced into *Agrobacterium tumefaciens* strain LBA4404 by heat shock method. Actively growing *Agrobacterium* cultures were prepared in LB medium containing 50 mg/L kanamycin and 25 mg/L rifampicin and incubated at 28°C for 16 to 18 hours until reaching an OD_{600} of 0.5 to 0.8. The callus tissues were pre-cultured for 48 hours on MS medium before being immersed in the bacterial suspension for 10 minutes with occasional gentle shaking. After co-cultivation on MS medium supplemented with 100 µM acetosyringone for 48 hours in the dark, the calli were transferred to a selection medium containing 50 mg/L kanamycin and 250 mg/L cefotaxime to eliminate excess bacteria. Transformed callus tissues were maintained on MS medium supplemented with 50 mg/L kanamycin to select for putative transformants. The cultures were subcultured every two weeks to promote growth and eliminate non-transformed tissues. Regeneration was attempted on MS medium containing 1.0 mg/L TDZ and 0.2 mg/L IAA and 0.2 mg/L GA under the same controlled environmental conditions. To confirm transgene expression, histochemical GUS staining was performed following Jefferson's (1987) protocol. Transformed and non-transformed callus tissues were incubated in GUS assay buffer containing X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) at 1, and 100 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, and 2 mM potassium ferricyanide. The samples were incubated at 37°C for 12–16 hours, followed by destaining with 70% ethanol.

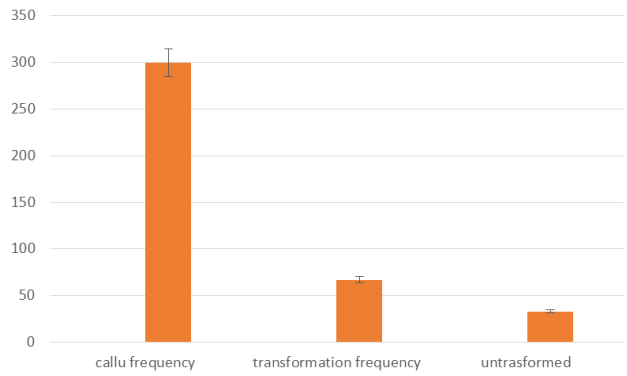

Fig. 1: Frequency Distribution of callus formation and transformation in Kashi Anmol variety of chili

Blue coloration in the transformed tissues confirmed GUS expression (Mahto et al., 2018). The transformation efficiency was calculated as the percentage of GUS-positive calli relative to the total number of infected calli. A total of 400 hypocotyl explants of *Capsicum annuum* were cultured for callus induction. Among them, 300 explants successfully formed callus, resulting in a callus induction frequency (CIF) of 75% **(**Aniel et al., 2010). Furthermore, the genetic transformation was performed on the induced calli, with 50 calli exhibiting successful transformation, corresponding to a transformation frequency (TF) of 16.67%, as given in Figure 1. These findings provide insights into optimizing callus induction and transformation efficiency in chili plant tissue culture. All experiments were performed in triplicate, and statistical analysis was conducted using ANOVA (Analysis of Variance) with $p < 0.05$ considered statistically significant. This study successfully established an optimized protocol for callus induction and genetic transformation in *Capsicum annuum* (Kashi Anmol variety) using Agrobacterium-mediated transformation. The achieved callus induction

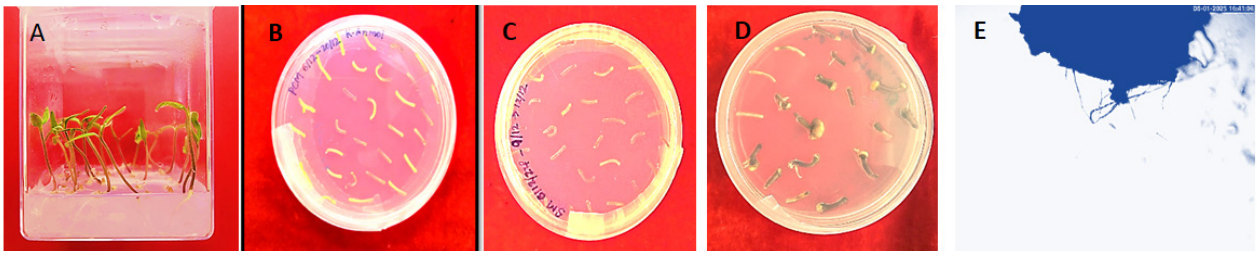

Fig. 2: A- 12 Days old Seedlings B- Co-cultivation of Hypocotyls on pre-culture media, C- Hypocotyls on selection media after co-cultivation, D- Callus formation and E- GUS assay in transformed callus

frequency of 75% and transformation efficiency of 16.67% highlight the potential of tissue culture-based genetic improvement in chili plants. The use of selective agents and histochemical GUS staining effectively confirmed transgene expression, validating the transformation approach as provided in Figure 2. Despite the relatively low transformation efficiency, the findings contribute to the ongoing efforts in developing stress-resistant and high-yielding chili varieties through genome editing.

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